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Three dimensional alginate-fucoidan composite hydrogel augments the chondrogenic differentiation of mesenchymal stromal cells

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ABSTRACT

Presence of sulfated polysaccharides like heparan sulphate has often been implicated in the regulation of chondrogenesis. However, recently there has been a plethora of interest in the use of non-animal extracted analogs of heparan sulphate. Here we remodeled alginate (1.5%) by incorporating fucoidan (0.5%), a natural sulphated polysaccharide extracted from seaweeds to form a composite hydrogel (Al-Fu), capable of enhancing chondrogenesis of human mesenchymal stromal cells (hMSCs). We confirmed the efficiency of fucoidan incorporation by FTIR and EDX analysis. Further, its ability to support hMSC attachment and chondrogenic differentiation was confirmed by SEM, biochemical glycosaminoglycan quantification, real-time quantitative PCR and immunocytochemical analyses of chondrogenic markers Sox-9, Collagen II, Aggrecan and COMP. Effect of Al-Fu hydrogel on hMSC hypertrophy was also confirmed by the downregulation of hypertrophic genes Collagen X and Runx2. This composite scaffold can hence be used as a cartilage biomimetic biomaterial to drive hMSC chondrogenesis and for other cartilage repair based therapies.

1. Introduction

Mesenchymal stromal cells (MSCs) are an attractive cell source used in the field of tissue engineering and regenerative medicine primarily due to their ability to differentiate into multiple lineages (Pittenger et al., 1999) thus minimizing the need to harvest cells from the damaged tissues (Chen, Rousche, & Tuan, 2006). These cells are readily expandable *in vitro* while maintaining their multi potential characteristics throughout expansion (Pittenger et al., 1999). In particular, to differentiate to chondrocytes, MSCs essentially require an organized microenvironment that supports cell–cell and cell-matrix interactions (Lee et al., 2008). Approaches commonly used to induce chondrogenesis of hMSCs include culturing them in micromass or pellet culture or encapsulating them in a three dimensional culture using alginate. However, the limiting factors of these approaches are the formation of necrotic cells in the pellet centre (Dashtdar et al., 2016; Vidal et al., 2008) and the upregulation of hypertrophic genes when seeded in a 3D construct made of alginate (Xu et al., 2008). Although hypertrophy was noted in cells encapsulated in alginate, it still remains the most commonly used scaffold mainly because it allows efficient transfer of nutrients and maintains cell proliferation and differentiation (Ma, Hung, Lin, Chen, & Lo, 2003). Alginate structure is known to consist of (1 → 4)-β-D-mannuronate (M) and α-L-guluronate (G) blocks found as linked co-polymers. These co-polymers are composed of repetitive G residues (GGGGGG), M residues (MMMMMM), and regions of alternating guluronic and mannuronic residues (GMGMGM) (Lee & Mooney, 2012). Studies show that though alginate provided the optimal template for the encapsulated cells to produce chondrogenic specific matrix, it lacked the microenvironment which mimic the native cartilage proteoglycan that helps to maintain the stability of the differentiated cells (Xu et al., 2008). Therefore, recent studies focus on remodeling the alginate hydrogel by incorporating components that mimic native cartilage extracellular matrix (ECM) niche like heparin sulfate or chondroitin sulfate (Little, Kulyk, & Chen, 2014). Besides directing differentiation, addition of heparin improved the biocompatibility of sodium alginate and also stabilized the supplemented growth factors (Jeon, Powell, Solorio, Krebs, & Alsberg, 2011). Moreover, heparin was also found to play an important role in the interaction with bioactive proteins associated with cell adhesion, proliferation and differentiation (Kim,

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Kim, & Tae, 2013). In addition to heparin, chondroitin sulfate was also found to stimulate chondrogenesis in cartilage tissue engineering (Kawamura, Funakoshi, Mizumoto, Sugahara, & Iwasaki, 2014). However, these sulfated polysaccharides were extracted from animal sources (Nagasawa & Uchiyama, 1984) and their use *in vivo* may carry the potential risk of prion, viral, or zoonoses contamination. Other means of alginate sulfation using chemical agents like sulphuric acid-carbodiimide (Freeman, Kedem, & Cohen, 2008) chlorosulfonic acid-formamide (Fan et al., 2011) and various SO_3/complexes (Kim, Lee, & Cho, 2003) has also been reported. However, their application *in vivo* is indecisive due to their chemical nature. Hence, there is a need for a natural, safer and relatively cost effective material with similar structural and functional properties of heparan/chondroitin sulfate.

Fucoidan, a sulfated polysaccharide extracted from marine brown algae, essentially contains fucose and uronic acids with a small proportion of galactose, xylose, arabinose, mannose, glucose and also possess sulfate groups which make it structurally similar to heparan and chondroitin sulfate. Fucoidan has a backbone made of $\alpha(1\rightarrow 3)$-L-fucopyranose residues or of alternating $\alpha(1\rightarrow 3)$ and $\alpha(1\rightarrow 4)$-linked L-fucopyranosyls and in both forms, these residues may be sulfate substituted (Ale, Mikkelsen, & Meyer, 2011). It has a number of important biological properties facilitating cartilage regeneration including water and nutrition absorption and inhibition of arthritis (Shu, Shi, Nie, & Guan, 2015). However, the role of fucoidan in inducing chondrogenesis of hMSCs, has to our knowledge not been addressed earlier.

In this study, we incorporated the sulfated polysaccharide fucoidan into alginate hydrogel to provide a favourable niche for inducing chondrogenesis of hMSC and preserving the chondrocytic phenotype. We hypothesized that modifying the alginate hydrogel with fucoidan can produce a chondro-inductive microenvironment, and this could enhance the chondrogenesis of mesenchymal stromal cells. We tested this hypothesis by evaluating its effect on the proliferation of the encapsulated hMSCs, gene expressions and protein synthesis of cartilage specific markers and compared it with hMSCs encapsulated in alginate. The effect of fucoidan on the regulation of hypertrophy, which is commonly seen in cells encapsulated in alginate hydrogel, was also evaluated.

2. Materials and methods

2.1. Isolation of mesenchymal stromal cells from human bone marrow

Human bone marrow samples were obtained from subjects who were undergoing fracture fixation involving the long bones. After providing the patient information sheet and explaining to the patients about the process of bone marrow collection, written informed consent was obtained from them. This study was approved by the Medical Ethics Committee of University of Malaya Medical Center (MECID.NO: 201412865). Human bone marrow samples were collected in sterile 3 mL BD Vacutainer blood tubes and transferred to the laboratory. hMSCs were separated using Ficoll-Paque (GE Healthcare,USA) centrifuged at 2200 rpm for 25 min, and the cells were cultured in complete medium (Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL of penicillin, 100 mg/mL of streptomycin, and 2 mM L-glutamine; all from Invitrogen, Canada) under standard conditions (37 °C, 5% carbon dioxide (CO_2) (v/v) in air). Thereafter, culture medium was changed every 3 days until it reached 80% confluency. The cells were then serially passaged. Passage three (P_3) cells were used in this experiment. Cells isolated were characterized using immunostaining for CD makers 44, CD105, CD34, CD45 and further by histological staining (procedure explained in the supplementary file). The results showed the cells were positive for CD44 and CD105. The cells were also able to differentiate to chondrocyte, adipocyte and osteoblasts lineages (Fig. S1).

2.2. Preparation and characterization of alginate-fucoidan (Al-Fu) and alginate (Al) hydrogel

Alginate-fucoidan (Al-Fu) composite hydrogel were prepared using 1.5% low viscosity alginate (320 kDa) (Al) composed of approximately 61% mannuronic and 39% guluronic acid (Sigma-Aldrich, USA) powder dissolved in 0.15 M sodium chloride and mixed with 0.5% fucoidan (72 kDa) (Fu) from Macrocystis pyrifera (≥85%) (Sigma-Aldrich). The concentration of fucoidan was fixed based on preliminary experiments done on hMSC proliferation and GAG production. The mixture was stirred at room temperature for 3 h. Al-Fu hydrogel were formed by exuding the alginate-fucoidan composite solution into 102 mM calcium chloride ($CaCl_2$) (Sigma Aldrich, USA) solution through 22G syringe needle. After Al-Fu solution was left polymerizing in calcium chloride solution for 10 min, the spherical hydrogels were washed twice with 0.15 M sodium chloride solution. Alginate (Al) hydrogel was prepared with 1.5% alginate powder using the same polymerization method as stated above. To evaluate the functional groups in the hydrogels by Fourier Transform Infrared Spectroscopy (FTIR) (Shimadzu FTIR-8400, Japan), the spherical hydrogel beads were subjected to overnight air drying. Spectral scanning was done in the range of 400–4000 cm^{-1}. Electron dispersive spectroscopy (EDX) was performed on Al-Fu hydrogel to confirm the elements present in the material using XMAX 20 EDS detector on Quanta FEG 250 ESEM (FEI, USA).

2.2.1. Encapsulation of hMSCs in alginate and alginate-fucoidan hydrogel

To prepare hMSC-alginate hydrogel and hMSC-alginate-fucoidan hydrogel, cells were first detached from culture flask using TrypLE Express (Invitrogen, USA). Detached passage 3 cells were pelletized using centrifuge (1500 rpm, 5 min) for cell counting. Cells were counted manually using a hemocytometer. 1 mL of Al solution (1.5% alginate dissolved in NaCl) and Al-Fu solution (1.5% alginate and 0.5% fucoidan dissolved in NaCl) was added to 1 million cells and mixed well to achieve a cell density of 1×10^6 cells/mL. The Al and Al-Fu suspensions with cells were introduced drop wise from a syringe with a size-22 needle into 100 mL of aqueous 102 mM $CaCl_2$ solution being stirred at 400 rpm. Hydrogels were removed with a spatula and incubated for 5 min free floating in a 60 mm petri dish containing 51 mM $CaCl_2$ to allow equilibration of $CaCl_2$ throughout the hydrogels. The hydrogel beads were then washed for 2 min in phosphate buffer solution before transferring to non-treated 24-well plates. hMSC encapsulated in Al and Al-Fu hydrogel were cultured in either normal medium (Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS)) or in chondrogenic medium supplemented with TGF β1. Medium was changed every 3 days.

2.3. Assessment of cell morphology

The morphology of cells in Al and Al-Fu hydrogel beads were determined using a scanning electron microscope (model JEOL JSM-6360, Japan). After 21 days of culturing the hMSCs encapsulated in Al and Al-Fu hydrogel in either normal medium or chondrogenic medium, the hydrogel beads were processed and mounted on a stub and then coated with carbon and gold (100 and 50 Å thickness respectively) sputter module in a vacuum evaporator in an argon atmosphere. The coated samples were then observed under a scanning electron microscope operated at 15 kV. Scanning elec-